# The Effects of a Nesprin-2-Giant Mutant on Nuclear Positioning and the Pathogenesis of Emery-Dreifuss Muscular Dystrophy

**Alexander McQuown**
**Advisor: G.W. Gant Luxton**
**Undergraduate Research Opportunities Program**
***Luxton Lab – Department of Genetics, Cell Biology, and Development – University of Minnesota***

## Abstract

Emery-Dreifuss muscular dystrophy is a muscle-wasting disease thought to be caused by improper nuclear positioning in muscle fibers. This nuclear positioning is mediated by the outer nuclear membrane (ONM) actin-binding protein nesprin-2-giant (N2G). A genetic mutation in *SYNE2*, the gene encoding N2G, has been identified in EDMD patients that results in an amino acid substitution (T6211M), but its role in EDMD is not understood. This study aims to construct wild type and mutant N2G cDNA constructs by assembling eleven smaller cDNA fragments and tagging the constructs with either green fluorescent protein (GFP) or the FLAG sequence for protein purification. GFP constructs will be used for *in vivo* analysis of how the mutation affects nuclear movement and N2G localization. Purified FLAG-tagged proteins will be used for *in vitro* studies to compare wild type and mutant thermal stability and actin affinity. During this UROP the eleven N2G cDNA fragments were assembled into three larger pieces. To determine if a protein lacking the transmembrane KASH domain would localize away from the ONM and be suitable for purification, GFP constructs encoding the N- and C-terminal domains of N2G and lacking the KASH domain were constructed. When expressed in U2OS cells, proteins lacking the KASH domain did not localize to the ONM, which is essential for purification of full length N2G. Future work will aim to finish assembling the three N2G cDNA fragments into the full gene to begin studying its role in EDMD pathogenesis.

## Introduction

- EDMD is a muscle-wasting disorder characterized by muscle contracture, progressive muscle wasting, and cardiomyopathy (1).
- EDMD is thought to be caused by improper formation of neuromuscular junctions (NMJ) (2).
- A proper NMJ requires the positioning of 4-8 synaptic nuclei below the synapse (3), which are not present in EDMD patients (4).
- Nuclear positioning in muscle tissue is mediated by the **LI**nker of the **N**ucleus and **C**ytoskeleton (LINC) complex (5)
- The LINC complex consists of ONM proteins (nesprins) and inner nuclear membrane (INM) proteins (SUNs) that physically connect the cytoskeleton to the nucleus.
- N2G is an ONM actin-binding protein that plays a role in the organization of organelles in skeletal muscle cells (6).
- EDMD patients show a mutation in *SYNE2*, the gene encoding N2G, resulting in the amino acid substitution T6211M (7).
- It is hypothesized that the T6211M mutation inhibits N2G-dependent nuclear positioning, leading to a lack of synaptic nuclei at the NMJ and the pathogenesis of EDMD.
- This study aims to create wild type and T6211M N2G constructs tagged with either GFP, for *in vivo* analysis of N2G subcellular localization and nuclear positioning, or tagged with FLAG, for purification and *in vitro* comparisons of wild type vs. T6211M stability and actin affinity.

## Methods & Materials

- The wounded fibroblast monolayer (WFM) system is used to study N2G dependent nuclear movement.
- In the WFM system, 3T3 fibroblasts are cultured in a monolayer and serum-starved. A wound is created on the monolayer and the serum component lysophosphatidic acid (LPA) is added, causing cells at the wound edge to polarize.
- During polarization a LINC complex-dependent nuclear movement occurs, moving the nucleus to the end of the cell opposite the wound edge and providing a robust assay for LINC complex-dependent nuclear movement.
- The WFM system has been used to show that mini-N2G, a truncated version of N2G containing the Calponin homology domains and KASH domain (Figure 1), is sufficient for nuclear movement in polarizing fibroblasts (8).
- Eleven ragments of N2G cDNA were previously cloned into plasmids and were present in the Luxton Lab, named A-J and core (Figure 1).
- During this UROP a GFP-mini-N2G construct was cloned in the pcDNA plasmid without the final 74 amino acids of the core fragment, which removed the transmembrane KASH domain (GFP-mini-N2G-ΔKASH).
- U2OS cells were transfected with this construct using Lipofectamine transfection reagent (Life Technologies) and analyzed using fluorescence microscopy to see if removal of the KASH domain affected localization of GFP-mini-N2G.
- The eleven N2G cDNA fragments were assembled into a total of three larger fragments using Gibson Assembly Cloning (New England Biolabs), named X, Y, and Z (Figure 2).

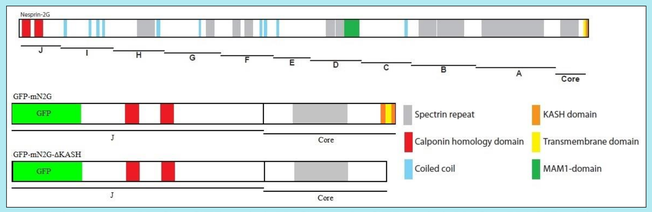


**Figure 1. N2G, GFP-mini-N2G, and GFP-mini-N2G-ΔKASH.** The Luxton Lab had cloned N2G cDNA into eleven smaller fragments, called A-J and core. The KASH domain was removed from GFP-mini-N2G.

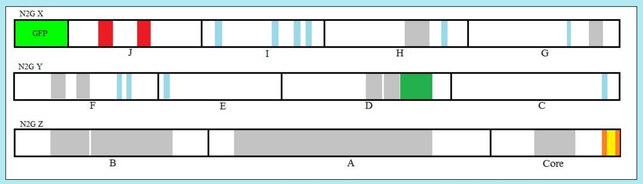


**Figure 2. N2G X, Y, and Z.** Using the Gibson Assembly cloning kit (New England Biosciences) the eleven cDNA fragments were assembled into three larger pieces of cDNA. Future work will assemble these pieces into full-length N2G.

## Results

- GFP-mini-N2G localized to the nuclear envelope and endoplasmic reticulum in transfected U2OS cells
- GFP-mini-N2G-ΔKASH was dispersed throughout the cytoplasm, as expected due to the removal of the transmembrane KASH domain

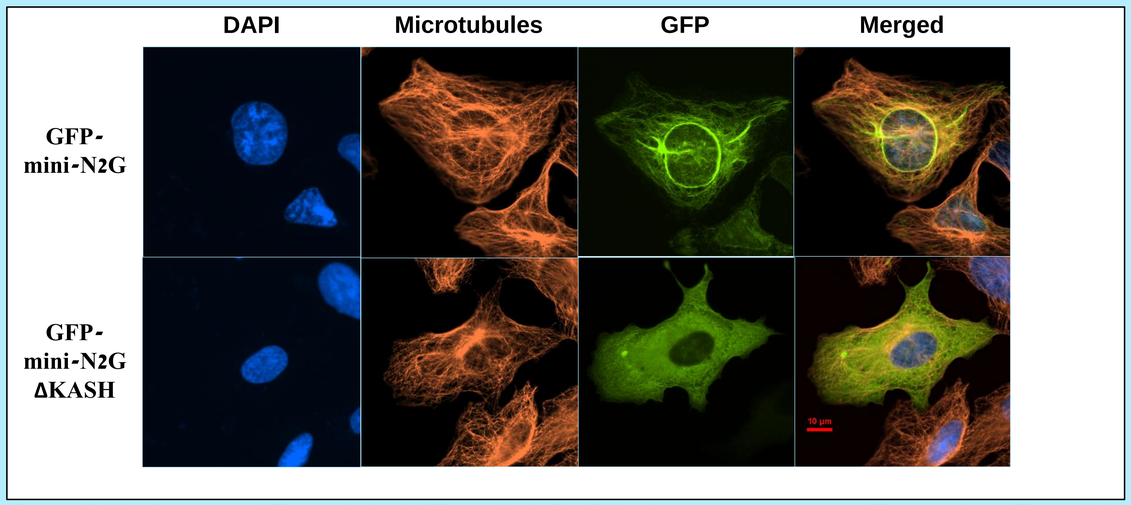


**Figure 3. U2OS cells expressing GFP-mini-N2G and GFP-mini-N2G-ΔKASH.** U2OS cells were transfected with GFP-mini-N2G and GFP-mini-N2G-ΔKASH and observed using fluorescence microscopy.

## Future Directions

- Assemble full-length N2G cDNA from N2G X, Y and Z (Figure 2)
- Create full-length GFP-N2G and FLAG-N2G-ΔKASH constructs
- Introduce the T6211M mutation to both constructs
- Microinject cDNAs encoding these constructs into the nuclei of wound edge NIH3T3 fibroblasts and observe localization and ability of each to rescue nuclear movement in N2G knockdown cells during cell polarization
- Transfect Sf9 insect cells with wild type and T6211M FLAG-N2G-ΔKASH and purify using the FLAG-affinity purification system (Sigma Aldrich)
- Perform circular dichroism spectroscopy on the purified proteins and observe and compare the thermal stability of wild type and T6211 N2G
- Perform actin affinity assays on wild type and T6211M N2G purified proteins

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